

AMENDMENTS TO THE SPECIFICATION:

On page 1, please amend the paragraph between lines 9 and 20 as follows:

Different cell processes common to multicellular organisms have been recently shown as dependent ~~of~~ on active targeted vesicular traffic, and therefore related to cell membrane dynamics and composition. The synaptic vesicles from the central nervous system, the vesicles called "exosomes" in the immune system, as well as virion budding from infected cells, are all examples of transmission of information pathways from one cell to another. Exosomes have been shown to originate from late endosomes or multi vesicular bodies (MVB) (Kobayashi et al., 1998). The exosome fusion with cells of the immune system induces stimulation of these cells (Kobayashi et al., 2002). The protein composition of these exosomes appears dependent on the cell, with some common components like tetraspan CD63 and CD82, which play an important role in the targeting of exosomes.

On page 2, please amend the paragraph between lines 3 and 18 as follows:

The inventors postulated that externalised *Dd* vesicles may participate in intercellular communication between *Dd* cells. To gain insight ~~in~~ into *Dd* vesicles function, vesicles were prepared either from the growth medium of *Dd* cells grown in presence of HO342, or from the starvation medium of the same

cells starved during 22 h in buffer without HO342. HO342, a bis-benzimidazole positively charged hydrophobic molecule that is an (A-T) specific DNA stain, exhibits fluorescence characteristics which are dependent on its environment and association state. These characteristics have been used to follow the state of HO342 inside *Dd* vesicles. The inventors have demonstrated the capacity of the dye-containing vesicles to mediate the transfer of the fluorescent dye to nuclei of naive living *Dd* cells, and more importantly to efficiently target the dye to the nuclei of human cells, either multidrug sensitive or resistant. Interestingly, the transfer efficiency of HO342 to the cells by *Dd* vesicles was found to be improved compared with liposomes containing the dye. These results identify *Dd* vesicles as mediators of intercellular communication.

On page 2, please amend the paragraph between lines 3 and 18 as follows:

Since other amoebae have been shown to shed extracellular vesicles, these results further indicate that non-pathogenic amoeba vesicles are useful as a new vehicle for the transport of a molecule of interest, in particular a therapeutic molecule, to eukaryotic cells, such as mammalian cells. Furthermore, these vesicles have a plasma membrane which composition is close to that of mammalian cells and deprived of virulence factors. This lack of pathogenic character makes it

possible for a safe use of the vesicles, while avoiding side effects such as septic ~~check~~ shock that may be encountered with vesicles originating from other micro-organisms, such as bacteria. Thus the vesicles according to the invention may find applications in vaccination, gene therapy, or any other therapeutic or prophylactic treatment where the transfer of a molecule of interest to a eukaryotic cell may be required.

Please amend the paragraph spanning from page 14 line 21 to page 15 line 2 as follows:

Figure 1 is a representation of fluorescence emission spectra analysis of Hoechst 33342 excited at 350 nm, free or complexed with DNA, lipids and SDS detergent. (a) The emission peak at 475 nm was of very low intensity (a.u.) for free HO342 (4-44 µg/ml in buffer A). (b) For HO342 (8 µg/ml) added to (Cp) DNA at a [bp]/[dye] ratio  $\approx 2$ , the emission peak at 450 nm was of a much higher intensity ( $\times 100$ ). HO342 associated with ~~LUV~~ large unilaminal vesicles ("LUV"), accounting for the higher second order diffusion at 700 nm, exhibited the same spectral characteristics. (c) In presence of SDS 2%, the emission spectra of HO342 (4-44 µg/ml), peaked at 470 nm, but the intensity was much higher ( $\times 170$ ) than the one of free HO342 in buffer A. (d) SDS 2% added to DNA-HO342 or lipid-HO342 complexes (as in b) was compared to SDS 2% added to HO342 8 µg/ml in buffer A. All emission spectra peaked around 470 nm but there was no intensity

increase for lipid-HO342 and only an increase  $\approx 2$  for DNA-HO342, compared to a factor of 170 for free HO342.

On page 22, please amend the paragraph between lines 7-18 as follows:

SDS-PAGE (12%) analysis performed under reducing conditions shows that both «12,000» and «105,000» C1 vesicles exhibited qualitatively comparable electrophoretic patterns with polypeptides of 97, 66-60, 45, and ~~35-30~~ 30-35 kDa. However, differences in signal intensities were observed for the 66-60 and 45 kDa polypeptides between the «12,000» and «105,000» (higher and lower intensity for the «12,000» C1 vesicles 60 and 45 kDa polypeptides, respectively, compared with the «105,000» C1 vesicles). When using gels 8% in acrylamide, a strong signal was detected at 205 kDa with both samples, in agreement with our previous data (Tatischeff et al., 1998). The «12,000» and «105,000» A1 vesicles, externalised by cells grown with HO342, exhibited electrophoretic patterns qualitatively similar to those observed for the control C1 vesicles.

On page 29, please amend the paragraph between lines 6 and 10 as follows:

Said vesicles are further isolated and concentrated and further contacted with K562 cells, as described above with the vesicles containing HO342, so as to assess whether K562 cells

acquired capacity to ~~synthesized~~ synthesize haemoglobin, i.e. whether the differentiated phenotype of the K562 cells is reverted by DIF-containing *Dd* vesicles.

On page 30, please amend the paragraph between lines 1 and 10 as follows:

The protein analysis of *Dd* vesicles indicated the presence of polypeptides of 97, 66-60, 45, and ~~35-30~~ 30-35 kDa. This has to be compared with the proteins associated with the purified *Dd* endocytic vesicles (Rodriguez-Paris et al., 1993; Nolta et al, 1994, Adessi et al., 1995). Among the major proteins, identified by sequence analysis, attention has to be paid, beside actin and a Rab 7-like GTPase, to the many subunits of the *D. discoideum* vacuolar H<sup>+</sup>-ATPase (p110, p66, p59, p41 and p29) and to a 34 kDa new cysteine protease (Adessi et al., 1995). Whether the polypeptides in the 60-66 kDa molecular mass region could ~~either~~ be the CD63 tetraspan requires a control by Western blot analysis with the specific antibodies.

Please amend the paragraph spanning from page 30 line 29 to page 31 line 31 as follows:

In the present study, the fluorescence properties of HO342 were used to demonstrate by spectrofluorimetry that the vesicles externalised from *Dd* cells grown in presence of HO342 actually contained the dye. That was true for both the «12,000»

and «105,000» vesicles, either purified directly from the cell growth medium (A1), or from the  $t_{22}$  starvation medium of the same cells (A2). The fluorescence characteristics of HO342 in buffer A (pH=6.8), as compared to HO342 bound to (Cp) DNA or to lipids, and/or to an anionic detergent, are in general agreement with previous studies on the photophysics of this dye (Gorner et al., 2001; Cosa et al., 2001; Shapiro et al., 1995; Shapiro et al., 1997). Indeed, a bathochromic shift in the dye absorption spectra was observed, as well as a blue-shifted fluorescence emission when HO342 was complexed ~~to~~ with DNA. These modifications were associated to an increase in the fluorescence intensity. Such spectral characteristics result from changes in the dipole moment of the molecule upon excitation or decay to the ground state and its interaction with the environment. Due to the structure of the bis-benzimide dye HO342 (FIG. 1a), its fluorescence deactivation is dependent on the configuration of the two benzimidazole units. In aqueous solvent ( $\approx$ pH 7), the binding between the two benzimidazole units is flexible and a radiationless decay of excitation arises from rotation along the bis-benzimide axis. In contrast, when HO342 is bound to DNA, the shifts in the absorption spectra and in the fluorescence emission, as well as the magnitude of the increase in the fluorescence quantum yield, are dependent on the location of the dye in the DNA structure. HO342 bound in the DNA small groove is only partly protected from the polar environment. Upon excitation, there is a charge

transfer process between the two benzimidazole subunits, resulting in a more planar configuration of the molecule. This should be the most predominant conformation in DNA, at a high [bp]/[dye] ratio, due to a rather tight binding of the dye in the DNA groove, which is responsible for the longer-lived component, *i.e.* for the blue shift and higher intensity of the HO342-DNA complex. However, at the low [bp]/[dye] ratio of our study, the average distance between two dye molecules should be  $\approx$  1-2 bp, where a steric hindrance could occur, favoring the twisted conformation at the expense of the planar one. The magnitude of the fluorescence increase is therefore lower than at a higher [bp]/[dye] ratio and remains very sensitive to the pH of the environment (Cosa et al., 2001).

Please amend the paragraph spanning from page 32 line 32 to page 33 line 10 as follows:

Finally, a video light-microscopy study of *Dd* cells, grown in the absence of HO342 and maintained in presence of the various HO342-containing vesicles, demonstrated the ability of these vesicles to target the DNA specific stain into the nuclei of living *Dd* cells. A similar study with human leukemic K562 cells, either ~~sensible~~ sensitive (s) or multidrug resistant (r), evidenced the capacity of the *Dd* HO342-containing vesicles to transfer the dye into the nuclei of the human cells, as well. The efficient targeting of HO342 to the nuclei of living cells is

then to be ascribed to the *Dd* vesicle-mediated delivery of free HO342. It is to be noticed that, in these conditions, only the nuclei of the cells appeared stained and not the membrane or any other cell compartment, i.e. the well-acknowledged specificity of HO342 for DNA was maintained at the cell level.